



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/US86/01318 <b>(22) International Filing Date:</b> 18 June 1986 (18.06.86) <b>(31) Priority Application Number:</b> 747,154 <b>(32) Priority Date:</b> 20 June 1985 (20.06.85) <b>(33) Priority Country:</b> US  <b>(71) Applicant:</b> THE SALK INSTITUTE FOR BIOLOGICAL STUDIES [US/US]; 10010 North Torrey Pines Road, La Jolla, CA 92037 (US).  <b>(72) Inventors:</b> ESCH, Frederick, Stephen ; 2929 Fire Mountain Drive, #63, Oceanside, CA 92507 (US). BAIRD, Andrew ; 7102 Teasdale, San Diego, CA 92122 (US). BOHLEN, Peter ; Huhnliackerweg 3, CH-8610 Uster (CH). GOSPODAROWICZ, Denis, Jerzy ; 215 Maywood Drive, San Francisco, CA 94127 (US). LING, Nicholas, Chai-Kwan ; 5324 Bloch Street, San Diego, CA 92122 (US).	<b>(74) Agents:</b> SCHUMANN, James, J. et al.; Fitch, Even, Tabin & Flannery, Room 900, 135 South LaSalle Street, Chicago, IL 60603 (US).  <b>(81) Designated States:</b> AT (European patent), BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).  <b>Published</b> <i>With international search report.</i>	
<b>(54) Title:</b> FIBROBLAST GROWTH FACTOR  <b>(57) Abstract</b>  Substantially pure basic fibroblast growth factor (bFGF), a 146 amino acid residue polypeptide, is produced. The amino acid residue sequence of bFGF is disclosed as well as a DNA chain encoding the polypeptide. By appropriately inserting a synthesized DNA chain into a cloning vector and using the cloning vector to transform cells, synthetic bFGF can be obtained from transformed cell lines, both prokaryotic and eukaryotic.		

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FIBROBLAST GROWTH FACTOR

The present invention is directed to fibroblast growth factor (FGF) and more particularly to basic FGF(bFGF) produced by synthetic methods, which will  
5 substantially enhance the availability of mammalian FGF.

BACKGROUND OF THE INVENTION

Both the brain and the pituitary gland have been known to contain mitogenic factors for cultured cells; however, until 1974, it was unclear what their  
10 relationship was with classical pituitary hormones, such as TSH, LH, FSH, GH and ACTH. In 1974, the identification in the pituitary gland of a growth factor called fibroblast growth factor (FGF) was reported which was shown to be distinct from pituitary hormones,  
15 Gospodarowicz, D. Nature, 249, 123-127 (1974). This growth factor is now known to have a MW of 16,415, is basic (a pI of 9.6), and is a potent mitogen for either normal diploid fibroblasts or established cell lines. Purification of an acidic brain FGF is described in U.S.  
20 Patent No. 4,444,760 (Apr. 24, 1984). Later studies confirmed that, in addition to fibroblasts, FGF is also mitogenic for a wide variety of normal diploid mesoderm-derived and neural crest-derived cells, including granulocytes, adrenal cortical cells,  
25 chondrocytes, myoblasts, corneal and vascular endothelial cells from either bovine or human origin, vascular smooth muscle cells, and lens epithelial cells. FGF was also shown to substitute for platelet-derived growth factor in its ability to support  
30 the proliferation of fibroblasts exposed to plasma-supplemented medium. Consistent with its ability to stimulate the proliferation of bovine and vascular endothelial cells, FGF has a similar activity in vivo on capillary endothelial cells; therefore, FGF is  
35 considered an angiogenic factor.

Mammalian fibroblast growth factor (FGF) can be purified using reverse-phase high performance liquid

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chromatography (RP-HPLC) and by the use of heparin-Sephareose affinity chromatography. Such methods of purifying FGF from mammalian tissue, such as brain and/or pituitary tissue, may be difficult to scale up to large scale production, and accordingly the production of pure FGF by synthetic methods should substantially enhance the availability of mammalian FGF.

#### SUMMARY OF THE INVENTION

The present invention provides pure basic fibroblast growth factor (bFGF) and a method by which it may be synthesized using recombinant DNA techniques or other suitable techniques. By bFGF is meant a 146 amino acid residue polypeptide having the sequence set forth hereinafter or an equivalent thereof. It appears most likely that in the native molecule none of the cysteine residues are disulfide bonded to each other, but that there may be bonding of one or more of the cysteine residues to free cysteine molecules. However, evidence for there being no internal disulfide-bonding between cysteine residues is not fully conclusive, and one or two pairs of cysteine residues may be internally bonded to each other. In any case, the present invention provides biologically active peptides, whether non-bonded or randomly bonded. Because bFGF is a relatively long-chain peptide, synthesis by a recombinant DNA technique is the synthetic method of choice, as opposed to standard chain elongation procedures involving stepwise addition of amino acid residues. Extraction and purification are possible but are not considered to be commercially feasible at the present time. Accordingly, a bFGF-encoding DNA chain is obtained, e.g., by oligonucleotide synthesis, and the synthetic DNA chain is inserted into a cloning vector, appropriately placed therein so as to ensure its expression when the recombinant cloning vector is introduced into an organism or cell line. Synthetic bFGF polypeptides which either have no internal

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disulfide bonds or which are randomly disulfide bonded exhibit biological activity.

Pharmaceutical compositions in accordance with invention include bFGF, a bFGF analog, biologically active fragments of bFGF or of analog bFGF, or nontoxic salts thereof, dispersed in a pharmaceutically acceptable liquid or solid carrier. Such pharmaceutical compositions can be used in clinical medicine, both human and veterinary, in acute or chronic administration for diagnostic or therapeutic purposes. bFGF is further useful in in vitro cell proliferation procedures. Also considered to be within the scope of the invention are peptides with additional segments added to either or both termini, such as those which arise from considerations of vector construction when the peptides are made using recombinant DNA techniques, providing that such terminal segments do not destroy the biological activity of the peptide.

## DETAILED DESCRIPTION OF CERTAIN PREFERRED EMBODIMENTS

20           The invention provides the first known pure  
mammalian bFGF, and the production thereof by synthetic  
methods. The nomenclature used to define the peptides  
is that specified by Schroder & Lubke, "The Peptides",  
Academic Press (1965), wherein in accordance with  
25       conventional representation the residue having the free  
alpha-amino group at the N-terminus appears to left and  
the residue having the alpha-carboxyl  
group at the C-terminus to the right. Where the amino  
acid residue has isomeric forms, it is the L-form of the  
30       amino acid that is represented. The invention provides  
peptides having the formula:

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15  
H-Pro-Ala-Leu-Pro-Glu-Asp-Gly-Gly-Ser-Gly-Ala-Phe-Pro-Pro-Gly-

16 17 18 19 20 21 22 23 24 25 26 27 28 29 30  
35 His-Phe-Lys-Asp-Pro-Lys-Arg-Leu-Tyr-Cys-Lys-Asn-Gly-Gly-Phe-

31 32 33 34 35 36 37 38 39 40 41 42 43 44 45  
Phe-Leu-Arg-Ile-His-Pro-Asp-Gly-Arg-Val-Asp-Gly-Val-Arg-Glu-

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46 47 48 49 50 51 52 53 54 55 56 57 58 59  
 Lys-Ser-Asp-Pro-His-Ile-Lys-Leu-Gln-Leu-Gln-Ala-Glu-Glu-Arg-  
 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75  
 Gly-Val-Val-Ser-Ile-Lys-Gly-Val-Cys-Ala-Asn-Arg-Tyr-Leu-Ala-  
 5 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90  
 Met-Lys-Glu-Asp-Gly-Arg-Leu-Leu-Ala-Ser-Lys-Cys-Val-Thr-Asp-  
 91 92 93 94 95 96 97 98 99 100 101 102 103 104 105  
 Glu-Cys-Phe-Phe-Phe-Glu-Arg-Leu-Glu-Ser-Asn-Asn-Tyr-Asn-Thr-  
 106 107 108 109 110 111 112 113 114 115 116 117 118 119 120  
 Tyr-Arg-Ser-Arg-Lys-Tyr-Ser-Ser-Trp-Tyr-Val-Ala-Leu-Lys-Arg-  
 10 121 122 123 124 125 126 127 128 129 130 131 132 133 134 135  
 Thr-Gly-Gln-Tyr-Lys-Leu-Gly-Pro-Lys-Thr-Gly-Pro-Gly-Gln-Lys-  
 136 137 138 139 140 141 142 143 144 145 146

15 Ala-Ile-Leu-Phe-Leu-Pro-Met-Ser-Ala-Lys-Ser-Y, wherein Y is  
 OH or NH<sub>2</sub>. It is uncertain whether the C-terminus of the  
 native molecule is amidated. For purposes of this  
 application, bFGF peptides should be considered to  
 constitute peptides having the 146 amino-acid-residue  
 sequence as well as biologically active fragments thereof.

20 From presently available evidence, it is most  
 likely that there is no internal disulfide-bonding  
 between cysteine residues of the chain. However, two of  
 the cysteine residues may be internally disulfide-bonded  
 to each other, and the residues at positions 25 and 69  
 25 are likely candidates for internal bonding. Although it  
 appears unlikely, disulfide bonding may occur between  
 two pairs of the cysteine residues. Also, one or more  
 of the cysteine residues, excluding any which are  
 involved in internal disulfide bonding, may be bonded to  
 30 free cysteine. The invention is intended to encompass  
 synthetically produced bFGF polypeptides in which the  
 cysteines are free or have random internal disulfide  
 bonds, i.e., between positions 25 and 69; 25 and 87; 25  
 and 92; 69 and 87; 69 and 92; 87 and 92; 25 and 69 plus  
 35 87 and 92; 25 and 87 plus 69 and 92; and 25 and 92 plus  
 69 and 87. A mixture of FGF peptides in which cysteine  
 residues are non-bonded or randomly bonded exhibits at

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least some biological activity. bFGF or "basic FGF" has a basic pI of 9.6 (in contrast to acidic FGF which has an acidic pI of about 5).

In any event, bFGF polypeptides produced by recombinant DNA techniques are inherently biologically active. This may be because the three-dimensional structure which bFGF assumes within cells is the structure recognized by the receptor. The three-dimensional structure which the molecule assumes through natural folding and through hydrophobic and hydrophilic interactions with aqueous media may promote desired bonding or non-bonding between cysteine residues. Also, enzymatic regulatory mechanisms within cells may help to ensure desired disulfide bonding or non-bonding, either by preventing bonding or by directing disulfide bonding between particular cysteine residues. Enzymes might also cleave "incorrect" bonding to enable the molecule to reorientate itself and assume the correct natural structure. Cysteine residues that are not internally bonded may be disulfide-bonded to free cysteine moieties. It may also be that the three-dimensional structure of the molecule is such that random bonding or non-bonding of cysteine residues either with each other or to free cysteines does not substantially affect the biological structure of the protein molecule.

To synthesize a protein having the bFGF amino acid residue sequence by recombinant DNA, a double-stranded DNA chain which encodes bFGF is synthetically constructed. The segment of the DNA chain that encodes bFGF is, of course, designed according to the genetic code; however, because of the degeneracy of the genetic code, a wide variety of codon combinations can be selected to form the DNA chain that encodes the product polypeptide. It is known that certain particular codons are more efficient for polypeptide expression in certain types of organisms, and the selection of codons preferably is made according to

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those codons which are most efficient for expression in the type of organism which is to serve as the host for the recombinant vector. However, any correct set of codons will encode product, even if slightly less efficiently. Codon selection may also depend upon vector construction considerations; for example, it may be necessary to avoid placing a restriction site in the DNA chain if, subsequent to inserting the synthetic DNA chain, the vector is to be manipulated using the restriction enzyme that cleaves at such a site. Also, it is necessary to avoid placing restriction sites in the DNA chain if the host organism which is to be transformed with the recombinant vector containing the DNA chain is known to produce a restriction enzyme that would cleave within the DNA chain.

In addition to the bFGF-encoding sequences, the DNA chain that is synthesized may contain additional sequences, depending upon vector construction considerations. Typically, the DNA chain is synthesized with linkers at its ends to facilitate insertion into restriction sites within a cloning vector. The DNA chain may be constructed so as to encode the bFGF amino acid sequences as a portion of a fusion polypeptide; and if so, it will generally contain terminal sequences that encode amino acid residue sequences that serve as proteolytic processing sites, whereby the bFGF polypeptide may be proteolytically cleaved from the remainder of the fusion polypeptide. The terminal portions of the synthetic DNA chain may also contain appropriate start and stop signals.

To assemble a bFGF-encoding DNA chain, oligonucleotides are constructed by conventional methods, such as procedures described in T. Manatis et al., Cold Spring Harbor Laboratory Manual, Cold Spring Harbor, New York (1982) (hereinafter, CSH). Sense and antisense oligonucleotide chains, up to about 70 nucleotide residues long, are synthesized, preferably on



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automated synthesizers, such as the Applied Biosystem Inc. model 380A DNA synthesizer. The oligonucleotide chains are constructed so that portions of the sense and antisense oligonucleotides overlap, associating with each other through hydrogen binding between complementary base pairs and thereby forming double stranded chains, in most cases with gaps in the strands. Subsequently, the gaps in the strands are filled in and oligonucleotides of each strand are joined end to end with nucleotide triphosphates in the presence of appropriate DNA polymerases and/or with ligases.

As an alternative to construction of a synthetic DNA chain through oligonucleotide synthesis, cDNA corresponding to bFGF may be prepared. A cDNA library or an expression library is produced in a conventional manner by reverse transcription from messenger RNA (mRNA) from a bFGF-producing cell line. To select clones containing bFGF sequences, hybridization probes (preferably mixed probes to accommodate the degeneracy of the genetic code) corresponding to portions of the FGF protein are produced and used to identify clones containing such sequences. Screening of the expression library with FGF antibodies may also be used, alone or in conjunction with hybridization probing, to identify or confirm the presence of bFGF-encoding DNA sequences in DNA library clones. Such techniques are taught, for example in CSH, supra.

The double-stranded bFGF-encoding DNA chain is constructed or modified with insertion into a particular appropriate cloning vector in mind. The cloning vector that is to be recombined to incorporate the DNA chain is selected appropriate to its viability and expression in a host organism or cell line, and the manner of insertion of the DNA chain depends upon factors particular to the host. For example, if the DNA chain is to be inserted into a vector for insertion into a

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prokaryotic cell, such as E. Coli, the DNA chain will be inserted 3' of a promoter sequence, a Shine-Delgarno sequence (or ribosome binding site) that is within a 5' non-translated portion and an ATG start codon. The ATG start codon is appropriately spaced from the Shine-Delgarno sequence, and the encoding sequence is placed in correct reading frame with the ATG start codon. The cloning vector also provides a 3' non-translated region and a translation termination site. For insertion into a eukaryotic cell, such as a yeast cell or a cell line obtained from a higher animal, the bFGF-encoding oligonucleotide sequence is appropriately spaced from a capping site and in correct reading frame with an ATG start signal. The cloning vector also provides a 3' non-translated region and a translation termination site.

Prokaryotic transformation vectors, such as pBR322, pMB9, Col E1, pCR1, RP4 and lambda-phage, are available for inserting a DNA chain of the length which encodes bFGF with substantial assurance of at least some expression of the encoded polypeptide. Typically, such vectors are constructed or modified to have a unique restriction site(s) appropriately positioned relative to a promoter, such as the lac promoter. The DNA chain may be inserted with appropriate linkers into such a restriction site, with substantial assurance of production of bFGF in a prokaryotic cell line transformed with the recombinant vector. To assure proper reading frame, linkers of various lengths may be provided at the ends of the bFGF-encoding sequences. Alternatively, cassettes, which include sequences, such as the 5' region of the lac Z gene (including the operator, promoter, transcription start site, Shine Delgarno sequence and translation initiation signal), the regulatory region from the tryptophane gene (trp operator, promoter, ribosome binding site and translation initiator), and a fusion gene containing these two promoters called the

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trp-lac or commonly called the Tac promoter are available into which the synthetic DNA chain may be conveniently inserted and then the cassette inserted into a cloning vector of choice.

5                    Similarly, eukaryotic transformation vectors, such as, the cloned bovine papilloma virus genome, the cloned genomes of the murine retroviruses, and eukaryotic cassettes, such as the pSV-2 gpt system (described by Mulligan and Berg, Nature 277, 108-114, 10 1979) the Okayama-Berg cloning system (Mol. Cell Biol. 2, 161-170, 1982), the expression cloning vector recently described by Genetics Institute (Science 228, 810-815, 1985), are available which provide substantial assurance of at least some expression of bFGF in the 15 transformed eukaryotic cell line.

                  A convenient way to ensure production of FGF or a polypeptide of a similar length is to produce the polypeptide initially as a segment of a gene-encoded fusion polypeptide. In such case, the DNA chain is 20 constructed so that the expressed polypeptide has enzymatic processing sites flanking the bFGF amino acid residue sequences. A bFGF-encoding DNA chain may be inserted, for example, into the beta-galactosidase gene for insertion into E. Coli, in which case, the expressed 25 fusion polypeptide is subsequently cleaved with proteolytic enzymes to release the bFGF from beta-galactosidase peptide sequences.

                  An advantage of inserting the bFGF-encoding sequence so that the bFGF sequence is expressed as a 30 cleavable segment of a fusion polypeptide, e.g., as the bFGF peptide sequence fused within the beta-galactosidase peptide sequence, is that the endogenous polypeptide into which the bFGF sequence is inserted is generally rendered non-functional, thereby 35 facilitating selection for vectors encoding the fusion peptide.

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Example 1

The structure of basic FRF of the bovine species was determined as follows:

Frozen bovine pituitaries were homogenized with a Waring blender for 5 minutes in 0.15 M ammonium sulfate (4 liter/kg tissue). The pH was then adjusted to 4.5 with HCl and the homogenate stirred vigorously for 2 hours. After centrifugation (18,000 x g, 30 minutes) the supernatant was retained, and 230g ammonium sulfate per liter of supernatant were added; the pH was adjusted to 6-6.5 with NaOH; and the precipitation was allowed to proceed for 15 hours. After centrifugation of the reaction mixture (18,000 x g, 30 min), the supernatant was retained; 300g ammonium sulfate were added to each liter of the supernatant; and then the mixture stirred well for two hours. After centrifugation of the reaction mixture (18,000 x g, 30 min), the pellet was retained, and the cumulative pellets from 3 kg starting tissue was dissolved in 200 ml distilled water and dialyzed against 20 liters of distilled water overnight. The pH of the dialyzed retentate was then adjusted to 6, and the solution was clarified by centrifugation (12,000 x g, 30 min). The dialyzed retentate constitutes a dialyzed extract.

Basic FGF was subsequently isolated from the dialyzed, clarified extract using three successive protocols; two of these employed conventional ion-exchange and reverse phase HPLC purification steps as described previously (P. Bohlen et al. Proc. Natl. Acad. Sci. USA 81, 5364-5368 (1984)). The third method utilized heparin-Sepharose affinity chromatography in a key purification step as detailed as follows in the order in which they were performed.

(A) CM-Sephadex (C50) ion-exchange chromatography.

A 7 X 9 cm column of carboxymethyl Sephadex (C50) was washed with 1 liter of 50 mM sodium phosphate,

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1.5 M sodium chloride, pH 6.0 and then equilibrated with 0.1 M sodium phosphate, pH 6.0. The dialyzed extract from 3 kg bovine pituitaries was loaded onto the column, and the column was washed sequentially with 0.1 M sodium phosphate, pH 6.0 containing a) no NaCl, b) 0.2 M NaCl and c) 0.65 M NaCl, allowing the OD<sub>280</sub> to reach a minimum value before initiating each new wash. Fractions of 18 ml were collected at 3 ml/min at 4°C and subjected to radioimmunoassay.

10 (B) Heparin-Sepharose chromatography.

The 0.65 M NaCl eluate from CM-Sephadex chromatography was loaded onto a 3 X 3 cm column of heparin-Sepharose (Pharmacia) previously equilibrated with 10 mM Tris-HCl, 0.6 M NaCl, pH 7.0 at room temperature. The column was then washed sequentially with 10 mM Tris-HCl, pH 7.0 containing a) 0.6 M NaCl and b) 1.1 M NaCl, allowing the OD<sub>280</sub> to reach a minimum value with each wash. The basic FGF was then eluted with a linear gradient in 10 mM Tris-HCl, pH 7.0 containing 100 ml 1.1 M NaCl and 100 ml 2 M NaCl. Fractions of 5 ml were collected at 0.8 ml/min and subjected to radioimmunoassay.

20 (C) Reverse phase liquid chromatography.

25 The basic FGF from heparin-Sepharose chromatography was pumped onto a Vydac C-4 (0.46 X 25 cm) reverse phase column (The Separations Group, Inc.) using a 0.1% trifluoroacetic acid (TFA)/acetonitrile solvent system (F. S. Esch et al. Methods in Enzymol. (ed. Conn, P.) 103, Academic Press, NY, pp. 72-89 (1983)) and eluted at 0.6 ml/min. with a 90 min. gradient of 23% to 35% acetonitrile. Fractions of 3 ml were collected at room temperature and subjected to radioimmunoassay.

35 In the above mentioned Radioimmunoassays (RIA) for basic FGF, antibodies were generated against a synthetic analog of the amino terminal sequence of basic

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FGF, [Tyr<sup>10</sup>]FGF(1-10) which is conjugated to bovine serum albumin, and were subsequently used to develop the radioimmunoassay for basic FGF, as described in A. Baird et al. Regulatory Peptides 10, 309-317 (1985).

5           Because it is not possible to quantitate unmodified cysteine by amino acid analysis, cysteine residues were modified either by reduction and alkylation with [<sup>14</sup>C]iodoacetamide (New England Nuclear) or oxidization with performic acid as indicated  
10 below. In either case, the FGF in 0.1% TFA/acetonitrile was dried in a 1.5 ml polypropylene microfuge tube in a Speed Vac vacuum centrifuge (Savant, Inc.) just prior to modification.

          The reduction and alkylation of cysteine  
15 residues was performed in order to radioactively label cysteine residues, making it possible to determine which fragments of subsequent cleavage reactions contain cysteine residues. The dried bFGF was dissolved in 0.1 ml deoxygenated 0.5M Tris-HCl pH 7.7, 10mM EDTA, 6M  
20 guanidine-HCl. Dithiothreitol was added to a final concentration of 5-10 mM, and the reduction was allowed to proceed at 37°C for 30 min. A 0.5-fold molar excess of [<sup>14</sup>C]iodoacetamide (24 mCi/mmol) over total  
25 sulfhydryl groups was added, and the incubation continued at 37°C for 60 min. in the dark. The alkylation was terminated by addition of a large excess of dithiothreitol over iodoacetamide, and the alkylated FGF was purified by reverse phase-high performance liquid chromatography.

30           Performic acid oxidation of cysteine converts cysteine to cysteic acid, and the cysteic acid content of the protein is measurable by amino acid analysis. Performic acid was generated by incubating 9 ml distilled formic acid with 1 ml 30% H<sub>2</sub>O<sub>2</sub> at room  
35 temperature in a tightly capped tube for 1 hour. 0.25 ml of this solution was employed to dissolve the dried FGF (5-15 nmoles), and the oxidation was permitted to

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continue at 0°C for 2.5 hours. Four lyophilizations from distilled water were employed to remove reaction by-products.

Basic FGFs (with cysteines modified by each method described above) were proteolytically and chemically digested to obtain fragments for further analysis, including sequence analysis. Prior to any digestion, the FGF was dried in a polypropylene microfuge tube in a Speed Vac vacuum centrifuge from volatile RP-HPLC solvents.

In order to obtain multiple, overlapping FGF fragments, three types of proteolytic digestions of bFGFs, with cysteines modified by each method described above, were performed as follows. The dried FGF (1-5 nmoles) was dissolved in 0.01 ml 0.5 M Tris-HCl pH 7.7, 10 mM EDTA, 6 M guanidine-HCl and then diluted to 1 ml with 1%  $\text{NH}_4\text{HCO}_3$ . Submaxillaris protease or chymotrypsin was added in a 1/50 (w/w) ratio while digestions with Staphylococcus aureus V8 employed a 1:35 (mol:mol) ratio of enzyme to substrate. Submaxillaris protease cleaves at the C-terminus of arginine; Staphylococcus aureus V8 cleaves at the C-terminus of glutamic acid; and chymotrypsin cleaves at the C-terminus of several amino acid residues having bulky aromatic or hydrophobic groups. Incubations were allowed to proceed overnight at 37°C.

Digestion with cyanogen bromide, which cleaves proteins at the C-terminus of Met, were performed on bFGFs, with cysteines modified by each method described above, as follows. The dried, alkylated FGF (5-6 nmoles) was dissolved with 0.05 ml 70% formic acid and reduced in a solution of 2.9 M N-methylmercaptoacetamide in 7% formic acid (R. Houghten et al. Methods in Enzymol. (eds. Hirs., C. & Timasheff, S.) 91, Academic Press, NY, pp. 549-559 (1983)) for 24 hours at 37°C. The alkylated, reduced FGF was purified by RP-HPLC, dried in a Speed Vac vacuum centrifuge and redissolved

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in 0.1 ml deoxygenated 70% formic acid. A 100-fold excess of cyanogen bromide was added and the incubation continued at room temperature in the dark overnight.

Reverse phase-high performance liquid  
5 chromatography purifications of modified bFGFs and their digestion fragments were accomplished using a Brownlee RP-300 reverse phase column (0.46 x 25 cm) and a 0.1% TFA/acetonitrile or a 0.1% heptafluorobutyric acid (HFBA)/acetonitrile solvent system (Esch et al. (1983)  
10 supra.).

Amino acid analyses and gas phase  
micro-sequencing of intact bFGF and its digestion fragments were carried out by methods previously described (P. Bohlen et al. Anal. Biochem. 126, 144-152  
15 (1982); F. S. Esch Anal. Biochem. 136, 39-47 (1984)). PhNCS- (<sup>14</sup>C)-carboxyamidomethylcysteine was identified during sequence analysis by liquid scintillation counting of the residues from the sequencer. The identification of cysteic acid in a given cycle was  
20 accomplished by comparison of the amino acid composition of the peptide and the remainder of its sequence as determined by Edman degradation. Carboxypeptidase Y was obtained from Pierce and utilized according to the manufacturer's recommendations. Carboxyl terminal  
25 analysis via tritium incorporation was accomplished as previously described (H. Matsuo et al. Protein Sequence Determination (ed., Needleman, S.B.) Springer-Verlag, NY, pp. 104-113 (1979)).

The highly efficient purification procedure,  
30 described above, permitted the rapid isolation of large quantities (about 30 to 60 nmoles per week) of highly purified basic FGF from bovine pituitaries. This source aided in the structural characterization effort. The heparin-Sepharose affinity chromatography purification  
35 step resulted in a several thousand-fold purification of two biologically active and basic FGF-immunoreactive mitogens, eluting at approximately 1.4 M and 1.95 M



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NaCl. A single step of RP-HPLC effected peptide homogeneity in each case. NaDodSO<sub>4</sub> PAGE yielded identical molecular weight estimates for both species, and gas phase micro-sequencing showed that both  
5 possessed identical amino terminal amino acid sequences through at least the amino-terminal 24 residues of each polypeptide. Pituitary extracts yielded approximately 15 times more of the mitogen eluting at 1.4 M NaCl than of the later eluting species, and hence, the former was  
10 selected for further structural characterization.

NaDodSO<sub>4</sub> PAGE suggested a molecular weight of 16,250  $\pm$  1000 for bovine pituitary basic FGF. Table I below shows the amino acid compositions obtained for the cationic mitogen from bovine brain and hypothalamus by  
15 R. R. Lobb et al., Biochem. 23, 6295-6299 (1984) as well as the compositional data obtained for basic FGF from bovine pituitary, all data being normalized for a 146 amino acid structure. The similarity of the compositions suggests that these structures are closely  
20 related, if not identical. In fact, basic FGF from bovine brain has been isolated, and it has been determined that its amino terminal sequence is identical to that of the pituitary-derived molecule.

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TABLE 1.

Amino Acid compositions of basic FGF from different bovine tissue

	Amino Acid	Brain <sup>a</sup>	Hypothalamus <sup>a</sup>	Pituitary	Basic FGF <sup>b</sup> (1-146)
5	Asx	13.7	13.0	12.4 $\pm$ 0.4	12
	Thr	5.1	4.9	3.9 $\pm$ 0.3	4
	Ser	10.0	10.0	9.4 $\pm$ 0.6	10
	Glx	13.2	14.2	14.1 $\pm$ 0.4 <sup>c</sup>	12
10	Pro	11.6	11.3	9.4 $\pm$ 0.6	10
	Gly	17.3	18.2	16.6 $\pm$ 0.6 <sup>c</sup>	15
	Ala	9.1	9.0	9.5 $\pm$ 0.4	9
	Cys	n.d.	n.d.	4.3 $\pm$ 0.2 <sup>d</sup>	4
	Val	5.8	5.7	5.9 $\pm$ 0.7	7
15	Met	2.4	2.4	1.6 $\pm$ 0.4	2
	Ile	3.2	3.1	3.4 $\pm$ 0.5	4
	Leu	12.6	12.9	13.4 $\pm$ 0.4	13
	Tyr	6.5	6.2	6.8 $\pm$ 0.4	7
	Phe	7.9	7.6	7.5 $\pm$ 0.2	8
20	His	3.2	3.2	2.4 $\pm$ 0.6	3
	Lys	13.7	13.5	13.9 $\pm$ 0.7	14
	Arg	10.8	10.4	11.6 $\pm$ 0.3	11
	Trp	n.d.	n.d.	0.4 $\pm$ 0.2	1

25 a Data from Lobb et al. supra. normalized for 146 amino acids.

b. Amino acid composition of basic FGF deduced from sequence analysis.

30 c Discrepancy between amino acid and sequence analysis data greater than that expected from statistical analysis.

d Cysteine was determined as cysteic acid after RP-HPLC purification of performic acid oxidized basic FGF.

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## Example 2

Using conventional methods, described in CSH, supra., a synthetic bFGF gene is constructed having the following formula:

```

5' AATTCATGCCAGCCCTACCAGAAGATGGGGGTCCGGGGCCTTCCCACCAGGG
3'   GTACGGTCGGGATGGTCTTCTACCCCCAGGCCCCGGAAGGGTGGTCCC
5
      CACTTCAAAGATCCAAAACGACTATATTGTAAAAACGGGGGGTTC
      GTGAAGTTTCTAGGTTTTGCTGATATAACATTTTTGCCCCCCAAG
      TTCCTACGAATCCACCCAGATGGGCGAGTAGATGGGGTACGAGAA
      AAGGATGCTTAGGTGGGTCTACCCGCTCATCTACCCCATGCTCTT
10
      AAATCCGATCCACACATCAAAC TACAAC TACAAGCCGAAGAACGA
      TTAGGCTAGGTGTGTAGTTTGATGTTGATGTTCTCGGCTTCTTGCT
      GGGGTAGTATCCATCAAAGGGGTATGTGCCAACCGATATCTAGCC
      CCCCATCATAGGTAGTTTCCCCATACACGGTTGGCTATAGATCGG
      ATGAAAGAAGATGGGCGACTACTAGCCTCCAAATGTGTAACCGAT
15
      TACTTTCTTCTACCCGCTGATGATCGGAGGTTTACACATTGGCTA
      GAATGTTTCTTCTTCGAACGACTAGAATCCAACAAC TATAACACC
      CTTACAAAGAAGAAGCTTGCTGATCTTAGGTTGTTGATATTGTGG
      TATCGATCCCGAAAATATTCCTCCTGGTATGTAGCCCTAAAACGA
      ATAGCTAGGGCTTTTATAAGGAGGACCATACATCGGGATTTTGCT
20
      ACCGGGCAATATAAACTAGGGCCAAAACCGGGCCAGGGCAAAAA
      TGGCCCGTTATATTTGATCCCGGTTTTTGGCCCGGTCCCGTTTTT
      GCCATCCTATTCCTACCAATGTCCGCCAAATCCTAAG      3'
      CGGTAGGATAAGGATGGTTACAGGCGGTTTAGGATTCAGCT 5'

```

25 Synthesis of a bFGF-encoding DNA chain is accomplished by synthesizing oligonucleotides on an applied B10 systems automatic synthesizer with overlapping complementary sequences.

30 The overlapping oligonucleotides are fused to form a double-stranded DNA chain, gaps being filled in with DNA polymerase and with T4 ligase. Immediately 5' of the FGF-encoding sequence in the sense strand is provided an ATG start signal, which results in an extraneous methionine being added to the N-terminus of the expressed polypeptide. Immediately 3' of the bFGF-encoding sequence is a stop signal. At the 5' end is a Eco RI overhang and at the 3' end is a Sal I

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overhang, whereby the synthetic DNA strand is directly insertable in the Eco RI and Sal I site of the plasmid pUC8, described by Vieira et al. Gene 14, 259-268 (1982). The DNA strand is annealed into the pUC8  
5 plasmid where it is under the control of the beta galactosidase promoter with the ATG start signal and the Shine Delgarno sequence retained in their natural orientation and association with the promoter.

The recombinant vector, designated bFGF, is  
10 transformed into the DH-1 strain of E. Coli by the calcium chloride procedure, CSH, supra.

The transformed E. Coli is cultured in L broth, and ampicillan-resistant strains are selected. Because the DNA chain was inserted into the plasmid in an  
15 orientation which could be expected to lead to expression of protein product of the DNA chain, the ampicillan-resistant colonies are screened for reactivity with antiserum raised against bFGF extracted from the pituitary. These colonies are screened by the  
20 immunological method of Healfman et al., Proc. Natl. Acad. Sci. USA 80, 31-35 (1983), and colonies reacting positively with bFGF antibody are further characterized. The cells are separated from their culture media are lysed, and their supernatent obtained. Supernatent from  
25 transformed cells is determined by RIA to be reactive with antibody raised against bFGF.

100 ml. of cell supernatent is obtained, and bFGF is purified therefrom using heparin-Sepharose as described above. Approximately 0.01 mg. of FGF,  
30 purified to upwards of 98% by weight of total protein, is produced.

The biological activity of the synthetic bFGF, which contains the extraneous N-terminal methionine residue, is tested for biological activity by the  
35 ability of the synthetic bFGF to stimulate the proliferation of adult bovine aortic arch endothelial cells in culture, as described in J. Cell Biol. 97,

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1677-1685 (1983). Briefly, cells (at passage 3-10) are seeded at a density of  $2 \times 10^3$  cells/dish on plastic tissue culture dishes and exposed to Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum. Test samples, at a dilution ranging from  $10^{-1}$  to  $10^{-3}$ , are added on day 0 and day 2 to the dishes. On day 4, triplicate dishes are trypsinized and counted in a Coulter counter. Background levels are ordinarily  $10^5$  cells/dish, while those exposed to optimal concentrations of the growth factor can contain as much as 5 to  $8 \times 10^5$  cells. For a potency assay, a log response curve was established. For this purpose, 10 microliter-aliquots of a dilution (ranging from  $10^{-1}$  to  $10^{-5}$ ) of the original solution made in 0.5% bovine serum albumin (BSA)/DMEM were added in triplicate.

The biological (mitogenic) activity of synthetic bFGF is substantially identical to natural, purified bFGF.

The superfluous N-terminal residue is removable by partial chemical digestion with cyanogen bromide or phenyl isothiocyanate followed by treatment with a strong anhydrous acid, such as trifluoroacetic acid. However, this process attacks internal Met residues, and while providing some bFGF having the natural protein structure, substantially reduces the total amount of biologically active protein.

### Example 3

Plasmid bFGF, amplified in one of the bFGF-producing E. Coli clones of Example 2, is isolated and cleaved with Eco RI and Sal I. This digested plasmid is electrophoresed on an agarose gel allowing for the separation and recovery of the amplified bFGF insert. The insert is inserted into the plasmic pYEp, a shuttle vector which can be used to transform both E. Coli and Saccharomyces cerevisiae yeast. Insertion of the synthetic DNA chain at this point assures that the DNA sequence is under the control of a promoter, in

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proper reading frame from an ATG signal and properly spaced relative to a cap site. The shuttle vector is used to transform URA3, a strain of S. cerevisiae yeast from which the orotate monophosphate decarboxylase gene is deleted.

The transformed yeast is grown in medium to attain log growth. The yeast is separated from its culture medium, and cell lysates are prepared. Pooled cell lysates are determined by RIA to be reactive with antibody raised against bFGF, demonstrating that a peptide containing bFGF peptide segments is expressed within the yeast cells.

The invention provides polypeptides and should make this important material available for biological and therapeutic use. The production of bFGF can be carried out in both prokaryotic and eukaryotic cell lines. While bFGF synthesis is easily demonstrated using either bacteria or yeast cell lines, the synthetic genes should be insertable for expression in cells of higher animals, such as mammalian tumor cells. Such mammalian cells may be grown, for example, as peritoneal tumors in host animals, and bFGF harvested from the peritoneal fluid.

Although the above examples demonstrate that bFGF can be synthesized through recombinant DNA techniques, the examples do not purport to have maximized bFGF production. It is expected that subsequent selection of more efficient cloning vectors and host cell lines will increase the yield of bFGF. Known gene amplification techniques for both eukaryotic and prokaryotic cells may be used to increase production of bFGF. Secretion of the gene-encoded polypeptide from the host cell line into the culture medium is also considered to be an important factor in obtaining synthetic FGF in large quantities.

FGF may also be synthesized using either classical synthesis and/or solid-phase synthesis to

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produce peptide segments of reasonable length. Such segments can then be appropriately linked to one another to create the desired 146-residue molecule.

Brain and pituitary FGF preparations, as reported earlier, are mitogenic for a wide variety of normal diploid cultured cells derived from tissue originating from the primary or secondary mesenchyme, as well as from neuroectoderm. These include rabbit chondrocytes, bovine granulosa and adrenal cortex cells, bovine corneal endothelial cells, capillary endothelial cells derived from bovine adrenal cortex and human umbilical endothelial cells.

bFGF peptides are useful biological materials for promoting in vitro growth of cultured cell lines, such as cell lines that have been transformed by recombinant DNA techniques to produce other useful polypeptides.

Furthermore, studies have shown that bFGF is capable of eliciting an angionenic response, for example, when implanted in the hamster cheek pouch or in the chick chorioallantoic membrane. Accordingly, substantially pure bFGF peptides have potential therapeutic applications.

Substantially pure FGF polypeptides can be routinely obtained having significantly higher purity than FGF polypeptides that are extracted from mammalian tissues, such as bovine pituitaries. FGF polypeptides constitute only very minor constituents of normal mammalian tissues and thus are present only in very impure form, relative to other native polypeptides also present. Recombinant DNA techniques, for example, can be used to generate organisms or cell lines that produce the heterologous polypeptide in significantly higher proportions relative to total protein, in the cellular material and/or their secretions, than the proportions at which native FGF polypeptides are present in mammalian tissue. Because the starting material from

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which such synthetic FGF polypeptides are isolated has a substantially greater concentration of the heterologous polypeptide, purification techniques can fairly simply produce more highly purified FGF polypeptide fractions.

5 Using isolation techniques such as those described hereinbefore, it is possible to routinely obtain bFGF polypeptides which are at least about 98% pure (by weight of total proteins) and which is herein referred to as substantially pure.

10 Substantially pure synthetic bFGF or the nontoxic salts thereof, combined with a pharmaceutically acceptable carrier to form a pharmaceutical composition, may be administered to mammals, including humans, either intravenously, subcutaneously, intramuscularly or

15 orally. The required dosage will vary with the particular condition being treated, with the severity of the condition and with the duration of desired treatment.

Such peptides are often administered in the form of pharmaceutically acceptable nontoxic salts, such

20 as acid addition salts or metal complexes, e.g., with zinc, iron or the like (which are considered as salts for purposes of this application). Illustrative of such acid addition salts are hydrochloride, hydrobromide, sulphate, phosphate, maleate, acetate, citrate,

25 benzoate, succinate, malate, ascorbate, tartrate and the like. If the active ingredient is to be administered in tablet form, the tablet may contain a binder, such as tragacanth, corn starch or gelatin; a disintegrating agent, such as alginic acid; and a lubricant, such as

30 magnesium stearate. If administration in liquid form is desired, sweetening and/or flavoring may be used, and intravenous administration in isotonic saline, phosphate buffer solutions or the like may be effected.

The peptides should be administered under the

35 guidance of a physician, and pharmaceutical compositions will usually contain the peptide in conjunction with a conventional, pharmaceutically-acceptable carrier.



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Although the invention has been described with regard to its preferred embodiments, which constitute the best mode presently known to the inventors, it should be understood that various changes and  
5 modifications as would be obvious to one having the ordinary skill in this art. For example, biologically active fragments can be employed instead of the entire 146-residue peptide, e.g. bFGF(24-120)-OH and bFGF(20-110)-NH<sub>2</sub>.

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What is claimed is:

1. Substantially pure basic fibroblast growth factor containing the amino acid sequence:

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
	Pro	Ala	Leu	Pro	Glu	Asp	Gly	Gly	Ser	Gly	Ala	Phe	Pro	Pro	Gly
5	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
	His	Phe	Lys	Asp	Pro	Lys	Arg	Leu	Tyr	Cys	Lys	Asn	Gly	Gly	Phe
	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45
	Phe	Leu	Arg	Ile	His	Pro	Asp	Gly	Arg	Val	Asp	Gly	Val	Arg	Glu
10	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
	Lys	Ser	Asp	Pro	His	Ile	Lys	Leu	Gln	Leu	Gln	Ala	Glu	Glu	Arg
	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75
	Gly	Val	Val	Ser	Ile	Lys	Gly	Val	Cys	Ala	Asn	Arg	Tyr	Leu	Ala
	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90
	Met	Lys	Glu	Asp	Gly	Arg	Leu	Leu	Ala	Ser	Lys	Cys	Val	Thr	Asp
15	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105
	Glu	Cys	Phe	Phe	Phe	Glu	Arg	Leu	Glu	Ser	Asn	Asn	Tyr	Asn	Thr
	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120
	Tyr	Arg	Ser	Arg	Lys	Tyr	Ser	Ser	Trp	Tyr	Val	Ala	Leu	Lys	Arg
	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135
20	Thr	Gly	Gln	Tyr	Lys	Leu	Gly	Pro	Lys	Thr	Gly	Pro	Gly	Gln	Lys
	136	137	138	139	140	141	142	143	144	145	146				
	Ala	Ile	Leu	Phe	Leu	Pro	Met	Ser	Ala	Lys	Ser				

2. bFGF according to Claim 1 wherein none of the four cysteine residues are internally disulfide bonded to each other.

3. bFGF according to Claim 1 wherein two of the four cysteine residues are internally disulfide bonded to each other.

4. bFGF according to Claim 1 wherein two pairs of cysteines are internally disulfide bonded to each other.

5. A method of producing a bFGF polypeptide comprising:

obtaining a DNA chain that encodes a polypeptide containing the following sequence:

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1 2 3 4 5 6 7 8 9 10 11 12 13  
 Pro-Ala-Leu-Pro-Glu-Asp-Gly-Gly-Ser-Gly-Ala-Phe-Pro-Pro-G-  
 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30  
 His-Phe-Lys-Asp-Pro-Lys-Arg-Leu-Tyr-Cys-Lys-Asn-Gly-Gly-Phe-  
 5 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45  
 Phe-Leu-Arg-Ile-His-Pro-Asp-Gly-Arg-Val-Asp-Gly-Val-Arg-Glu-  
 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60  
 Lys-Ser-Asp-Pro-His-Ile-Lys-Leu-Gln-Leu-Gln-Ala-Glu-Glu-Arg-  
 10 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75  
 Gly-Val-Val-Ser-Ile-Lys-Gly-Val-Cys-Ala-Asn-Arg-Tyr-Leu-Ala-  
 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90  
 Met-Lys-Glu-Asp-Gly-Arg-Leu-Leu-Ala-Ser-Lys-Cys-Val-Thr-Asp-  
 91 92 93 94 95 96 97 98 99 100 101 102 103 104 105  
 Glu-Cys-Phe-Phe-Phe-Glu-Arg-Leu-Glu-Ser-Asn-Asn-Tyr-Asn-Thr-  
 15 106 107 108 109 110 111 112 113 114 115 116 117 118 119 120  
 Tyr-Arg-Ser-Arg-Lys-Tyr-Ser-Ser-Trp-Tyr-Val-Ala-Leu-Lys-Arg-  
 121 122 123 124 125 126 127 128 129 130 131 132 133 134 135  
 Thr-Gly-Gln-Tyr-Lys-Leu-Gly-Pro-Lys-Thr-Gly-Pro-Gly-Gln-Lys-  
 136 137 138 139 140 141 142 143 144 145 146  
 20 Ala-Ile-Leu-Phe-Leu-Pro-Met-Ser-Ala-Lys-Ser,  
 inserting said DNA chain into a cloning vector  
 in proper relationship to DNA sequences which promote  
 expression of said encoded polypeptide,  
 transforming an organism or cell line with said  
 25 cloning vector having said inserted DNA chain,  
 culturing said transformed organism or cell  
 line, and  
 obtaining said bFGF polypeptide produce thereby.  
 6. A method according to Claim 5 wherein said  
 30 DNA chain is synthesized by stepwise construction of  
 oligonucleotides with overlapping complementary  
 sequences.  
 7. A method according to Claim 5 wherein said  
 DNA chain is obtained by producing a cDNA clone library  
 35 from a bFGF-producing cell line, determining a clone  
 having said DNA chain and excising said DNA chain from  
 said cDNA clone.

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8. A method according to Claim 5 wherein said organism is prokaryotic.

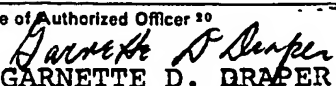
9. A method according to Claim 5 wherein said organism or cell line is eukaryotic.

5 10. A microorganism transformed with a cloning vector according to Claim 5 capable of expressing bFGF.

11. A microorganism according to Claim 10 which is a strain of E. Coli or a strain of S. cerevisiae yeast.

# INTERNATIONAL SEARCH REPORT

International Application No **PCT/US86/01318**

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all) <sup>3</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC		
INT CL 4 C07K 13/00, 15/04; C12P 21/00		
U.S. CL. 530/399, 350, 324; 435/68		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>4</sup>		
Classification System	Classification Symbols	
U.S.	530/399, 324, 350, 827, 825; 435/68	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>5</sup>		
Search in STN System; File CA and File Biosis for: 1) Basic-Fibroblast Growth Factor and 2) amino acid sequence thereof 3) RNA, DNA Recombinant or Unglycosylated and FGF.		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT</b> <sup>14</sup>		
Category <sup>6</sup>	Citation of Document, <sup>10</sup> with indication, where appropriate, of the relevant passages <sup>17</sup>	Relevant to Claim No. <sup>18</sup>
Y	Journal Cellular Biochemistry, Vol. 21, Issued 1983, (LEMMON), "Purification and Partial Characterization of Bovine Fibroblast Growth Factor", pages 195-208.	1-4
Y	Proc. Natl. Acad. Sci., Vol. 75, Issued 1978 (WESTALL), "Brain-derived Fibroblast Growth Factor: Identity with a Fragment of the Basic Protein Myelin", pages 4675-78.	1-4
Y	Journal Biol. Chem., Vol. 257, Issued 1982 (GOSPODAROWICZ), "Purification in High Yield of Brain Fibroblast Growth Factor by Preparative isoelectric Focusing at pH 9.6", pages 12266-78.	1-4
Y	Journal Biol. Chem. Vol. 257, Issued 1982, (GAMBARINI), "Purification and Partial Characterization of an Acidic Fibroblast Growth Factor From Bovine Pituitary", pages 9692-97.	1-4
<div style="display: flex; justify-content: space-between;"> <div style="width: 48%;"> <p><sup>15</sup> * Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 48%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p> </div> </div>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search <sup>7</sup>	Date of Mailing of this International Search Report <sup>8</sup>	
05 SEPTEMBER 1986	12 SEP 1986	
International Searching Authority <sup>1</sup>	Signature of Authorized Officer <sup>10</sup>	
ISA/US	 <b>GARNETTE D. DRAPER</b>	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, <sup>16</sup> with indication, where appropriate, of the relevant passages <sup>17</sup>	Relevant to Claim No <sup>18</sup>
P	Proc. Natl. Acad. Sci., Vol. 81, Issued 1984, (BOHLEN), "Isolation and Partial Molecular Characterization of Pituitary Fibroblast Growth Factor", pages 5364-68.	1-11
P	Proc. Natl. Acad. Sci., Vol 81, Issued 1984, (GOSPODAROWICZ), "Isolation of Brain Fibroblast growth Factor by Heparin-Sepharose Affinity Chromatography", pages 6963-67.	1-11
P	Proc. Natl. Acad. Sci., Vol. 82, Issued 1985 (ESCH), "Primary structure of bovine basic Fibroblast growth factor and comparison with the amino-terminal sequence of bovine brain acidic FGF", pages 6507-11.	1-11
P	Biochem. Biophys. Res. Comm., Vol. 133, Issued 1985, (ESCH), "Primary Structure of bovine Brain Acidic FGF", pages 554-62.	1-11
P	EMBO Journal, Vol. 4, Issued 1985, (BOHLEN), "Acidic FGF from bovine brain: amino-terminal sequence and comparison with basic FGF", pages 1951-56.	1-11
P	Biochem., Vol. 24, issued 1985, (BAIRD), "Retina and Eye-Derived Endothelial Cell Growth Factors", pages 855-60.	1-11
P	Biochem. Biophys. Res. Comm., Vol. 128, Issued 1985, (GASPODAROWICZ), "Fibroblast Growth Factor in the Human Placenta", pages 554-62.	1-11
P	Biochem. Biophys. Res. Comm., Vol. 135, Issued 1985, (GIMENEZ-GALLEGO), "Human Brain-Derived Acidic and Basic Fibroblast Growth Factors", pages 541-48.	1-11
P	Fed. Proc., Vol. 45, Issued 1986, (MOSCATELLI), "Basic Fibroblast Growth Factor-like Molecules Are produced by a Variety of Cultured Cells", page 1718.	1-11

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